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Selective depletion of intracellular oncogenic proteins is a potentially powerful tool for the treatment of breast cancer. This is usually achieved by genetic manipulation of the target gene using procedures such as gene disruption, antisense or ribozyme technologies. We now propose an alternative approach in which an oncogenic protein is specifically targeted for intracellular degradation. In order to do this we will take advantage of the permeability properties of the third helix of the antennapedia protein. This will be used to deliver a small trifunctional peptide consisting of a target protein binding peptide and a peptide designed to interact with the E2 class of ubiquitin conjugating enzymes. In this way the ubiquitin-conjugating machinery will be selectively recruited to the target protein which should then be degraded by the proteosome. We will use the cytoplasmic signaling molecule β-catenin as a model system since its oncogenic activity is thought to be regulated at the level of protein stability and we have established that it is normally targeted for ubiquitination and proteosomal degradation. Mutations of β-catenin which increase its protein stability are oncogenic. The β-catenin binding peptide will be based on the region of the tumor suppressor protein APC which constitutively binds β-catenin.

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Introduction

Selective depletion of intracellular oncogenic proteins is a potentially powerful tool for the treatment of breast cancer. This is usually achieved by genetic manipulation of the target gene using procedures such as gene disruption, antisense or ribozyme technologies. We now propose an alternative approach in which an oncogenic protein is specifically targeted for intracellular degradation. In order to do this we will take advantage of the permeability properties of the third helix of the antennapedia protein. This will be used to deliver a small trifunctional peptide consisting of a target protein binding peptide and a peptide designed to interact with the E2 class of ubiquitin conjugating enzymes. In this way the ubiquitin-conjugating machinery will be selectively recruited to the target protein, which should then be degraded by the proteosome. We will use the cytoplasmic signaling molecule β-catenin as one model system since its oncogenic activity is thought to be regulated at the level of protein stability and we have established that it is normally targeted for ubiquitination and proteosomal degradation. Mutations of β-catenin that increase its protein stability are oncogenic. The β-catenin binding peptide will be based on the region of the tumor suppressor protein APC that constitutively binds β-catenin. A second target will be ErbB-2 a tyrosine kinase strongly associated with breast cancer. The ErbB-2 binding peptide will consist either of the last three armadillo repeats of β-catenin known to constitutively bind ErbB-2 or the SH2 domain of grb2 which can only bind tyrosine phosphorylated ErbB-2. Generally, the work proposed is of great significance to the treatment of breast cancer. More specifically, proof of the principle that direct targeting of oncogenic proteins for intracellular degradation inactivates their transforming capabilities could lead to the development of novel therapuetic strategies based on this approach.

Revised Statement of Work

The reviewer of year 2 of the Progress Report of my grant DAMD-98-1-8089 suggested that I provide a revised statement of work to more adequately incorporate the new data on I kappa B kinase. I have submitted this new SOW, which was recently approved. Since this grant was first submitted we have gathered important new information relating to the sites on β-catenin that regulate its phosphorylation, ubiquitination and degradation. In particular we found that IKKα, a kinase thought previously to only phosphorylate IκB proteins to regulate NF kappa B, is a potent and important kinase in the regulation of β-catenin phosphorylation and ubiquitination. In fact, our results point to IKKα rather than GSK-3β as the key enzyme in this process. This work was summarized in the original Progress Report. These results are extremely relevant to the present grant since they raise the possibility that sites other than, or in addition to, those we originally proposed to utilize in the construction of targeting vectors could be important. In addition, the results raise the possibility that vectors designed to target β-catenin may also interfere with NF-kappa B signaling. Clearly it is important that we understand the details of this important relationship. The revised Statement of Work now reflects this broadening of scope (see below). In addition we have applied for a non-funded extension of the grant so that we can complete the new experiments.

Year 1

a) Construction of at least 6 expression vectors containing erbB2 and β-catenin targeting, antennapedia and cyclin B destruction box fusion constructs. These will consist of vectors containing several different erbB-2 and β-catenin targeting sequences as well as cyclin B destruction boxes of various lengths and sequence. Our aim is to ascertain the minimum size of the final trifunctional peptide product that is effective in targeting erbB-2 and β-catenin for degradation.
b) Co-express the vectors with erbB-2 or stable β-catenin (S37A mutant), investigate interaction with β-catenin.

Year 2

a) Investigate the role of the IKK complex in the regulation of β-catenin protein levels and signaling.
b) Continue experiments with expression vectors. Monitor β-catenin ubiquitination and degradation.
c) Adherex to make membrane permeant peptides and/or production of recombinant peptides.
d) Test the ability of peptides to enter cell cytoplasm and/or nucleus.

Years 3 and 4

a) Identify IKK phosphorylation sites on β-catenin.
b) Characterize the mechanism of IKK regulation of β-catenin signaling activity.
c) Redesign the targeting peptides based on this information.
d) Test the activity of the redesigned peptides on β-catenin for ubiquitination and degradation in vitro.
e) Test the ability of peptides to inhibit growth and colony formation of β-catenin-transformed cells.

Body:

In the first annual report we described in detail the progress we had made in constructing targeting vectors for ErbB-2. Briefly the key research accomplishments of the first 12 months were:

1) A number of different targeting vectors have been constructed.

2) The targeting constructs have been transfected into three different cells of varying ErbB-2 status

3) Two of the constructs yield protein products of the predicted size indicating that the recombinant peptides are stable and can be expressed at relatively high levels.

4) The constructs were detected with an antibody directed at the FLAG tag indicating that it is accessible and does not interfere with protein production.

In the second year of funding we concentrated on β-catenin. Since this grant was first submitted we gathered important new information relating to the sites on β-catenin that regulate its phosphorylation, ubiquitination and degradation. In particular we found that IKKα, a kinase thought previously to only phosphorylate IκB proteins, is a potent and important kinase in the regulation of β-catenin phosphorylation and ubiquitination. In fact, our results point to IKKα rather than GSK-3β as the key enzyme in this process (see report for year 2). These results are extremely relevant to the present grant since they raise the possibility that sites other than, or in addition to, those we originally proposed to utilize in the construction of targeting vectors could be important. This work was summarized in the previous report.

Results for year 3

In the third year we continued our work on the Wnt/β-catenin IKK pathways. This work was carried out collaboration with the laboratories of Richard Gaynor at UT Southwestern and Dr. Richard Pestell at Alb Einstein. We show a requirement for IKKα in PI3K-dependent induction of cyclin D1 and using cells from m homozygously deleted of the *cyclin D1* gene, show a requirement for cyclin D1 in PI3K-dependent cellu proliferation. Since IKKα but not IKKβ induced cyclin D1 and β-catenin/Tcf activity, these studies indicate t relative abundance of IKKα and IKKβ alters their substrate and signaling pathway specificity. The divergent effe of IKKα and IKKβ on β-catenin/Tcf signaling suggest the IKK complex may play a key role in coordinating t Wnt pathway. The demonstration that IKK interacts directly with and phosphorylates β-catenin to regulate t

expression of growth promoting genes such as cyclin D1 is clearly very important in breast cancers that associated with cyclin D1 overexpression (ref).

Cyclin D1 is required for PI3K-dependent S-phase entry in primary cells.

Activation of phosphotidyl inositol (PI) 3'-kinase (PI3K) mediates signaling induced by a number of growth fact and tumor promoters and is required for mitogenic stimulation by specific growth factors during the G_1-S phase the cell-cycle [24, 27]. The role of PI3K in serum-induced cyclin D1 expression was examined in mouse embr fibroblasts (MEFs). In wildtype MEFs, cyclin D1 protein levels were elevated by 3 hrs after serum stimulation a the PI3K inhibitor, LY294002, abrogated the induction (Fig. 1a). Total ERK levels were unchanged and cyclin was not induced by serum, however LY294002 reduced cyclin E levels by 30%. C*yclin D1*$^{-/-}$ MEFs of identi passage (Fig. 1a) showed similar levels of total ERK and a delayed decrease in cyclin E levels (60%) 12 hrs af serum addition (not shown). Western blot analysis to detect the phosphorylation status of Akt showed an inducti of Akt phosphorylation at 15 min and 3 hrs after serum stimulation and a 60% reduction with LY294002 at 2 hr both *CD1*$^{-/-}$ and *CD1*$^{+/+}$ MEFs (not shown). In wildtype MEFs, serum induced entry into the DNA synthetic pha with the S-phase fraction increasing from 10% to 54% (Fig. 1b). LY294002 reduced the S-phase proportion fr 23% to 7% at 24 hrs, and from 54% to 5% at 48 h, indicating that serum-induced DNA synthesis is PI3K -depend in MEFs. Serum induced DNA synthesis was reduced to 14% in the *cyclin D1*$^{-/-}$ MEFs (Fig. 1b). Thus cyclin D1 required for PI3K-dependent induction of DNA-synthesis by serum. To determine the role of PI3K in apopto mediated by serum deprivation annexin V staining and sub G_1 analysis was performed on the MEFs. In the ba state, *cyclin D1*$^{-/-}$ MEFs exhibited a 5-fold greater level of annexin V staining, indicating increased apoptosis which was rescued by serum (Fig. 1c). LY294002 did not affect the level of cellular apopotosis in either wt *cyclin D1*$^{-/-}$ MEFs as determined by either annexin V staining (Fig. 1c) or sub G_1 analysis (not shown). Simi analyses were performed on serum-induced DNA synthesis in 3T3 cells derived from the *CD1*$^{+/+}$ and *cyclin D1* MEFs. Serum-induced S-phase entry was inhibited by LY294002 in *CD1*$^{+/+}$, whereas S-phase was poorly induc in the *cyclin D1*$^{-/-}$ 3T3 cells and LY294002 had no effect (Fig. 1d). Together these findings suggest serum-induc DNA synthesis requires PI3K and that a substantial proportion of PI3K-dependent induction of DNA synthe requires cyclin D1. Other mechanisms of S-phase entry must exist in the *cyclin D1*$^{-/-}$ cells, which are lik dependent on cyclin E. PI3K signaling does not play a dominant role in the increased apopotosis induced by ser deprivation in *cyclin D1*$^{-/-}$ MEFs.

PI3K-induction of cyclin D1 is dependent upon IKKα.

Type 1 PI3K is a heterodimeric holoenzyme, consisting of regulatory (p85) and catalytic (p110) subuni that was initially identified through its role in Src-mediated transformation. Since the induction of cyclin D1 prot levels by serum was PI3K sensitive, and because activation of PI3K and Akt plays a key role in DNA synthesis prostate cancer cells [25, 26], we examined whether the cyclin D1 promoter was directly induced by constitutiv active PI3K (p110α-CAAX). Oncogenic forms of p110α and p85 have been identified and expression constitutively active PI3K triggers DNA synthesis [24,, 29]. We examined the role of PI3K in the PTEN containi prostate cancer cell line DU145. Cyclin D1 abundance was induced 4-fold by serum addition and the induction reduced 45% by LY294002 (data not shown). The cyclin D1 promoter (-1745 CD1LUC) was induced 2.5-fold p110α-CAAX while the kinase dead (p110α-CAAX KD) mutant was inactive (Fig. 2a). The consitutively acti p110α-K227E mutant induced cyclin D1 2.2-fold (Fig. 2a). Cyclin D1 protein levels in cells transfected with t p110α-CAAX vector were induced 3-fold (not shown). The PI3K responsive c-*fos* promoter was induced 10-f (data not shown), but the cyclin E and cyclin A promoters were not (Fig. 2b), suggesting the induction of cyclin is not an indirect effect of PI3K activity on DNA-synthesis and is promoter specific. Since cryptic activati sequences, including AP-1, have been identified in several expression vectors, we examined the empty lucifer reporter pA3LUC in which the cyclin D1 promoter was cloned and found that pA3LUC was not induced wh

pGL$_3$LUC was induced 3-fold by p110α-CAAX (Fig. 2b). Cyclin D1 promoter activation by p110α-CAAX inhibited by either LY294002 (Fig. 2c) or Wortmannin (not shown), by dominant negative mutants of the PI regulatory subunit (Fig. 2d), an N17Rac mutant and a kinase inactive dominant negative Akt (Akt K179M) but by wt Akt (Fig. 3d). The finding that Rac1-N17 blocked PI3K-induced activity is consistent with previous stud [30]. Since activating mutations of Rac1 induce both NF-κB activity and the *cyclin D1* gene through an NF-κ dependent pathway [31], we examined the possibility that NF-κB activity may play a role in PI3K induction of cyc D1 using dominant IκB inhibitor [CMV-IκB]. CMV-IκBαSr inhibited p110α-CAAX-induced activation of cyc D1 (Fig. 2d) but did not inhibit c-*fos* LUC activity (not shown). The p38 MAPK inhibitor SB203580, the E inhibitor PD98059 and rapamycin did not effect p110α-induced D1 activity (not shown). Both the domin negative and kinase dead IKKα reduced PI3K-induced cyclin D1 promoter activity and basal promoter activity i dose-dependent and promoter specific manner (Fig. 2e). Since the inhibition by IκBαSr suggested that p110 CAAX induction of cyclin D1 may involve IKK/NF-κB activity, we assessed the effect of p110α-CAAX on the κB-responsive reporter 3κBLUC. p110α-CAAX induced 3κBLUC activity 3-fold which was inhibited by eit LY294002 (Fig. 2f) or the dominant inhibitor CMV-IκB Sr (not shown).

IKKα, but not IKKβ induces cyclin D1 through β-catenin/Tcf.

The studies described above indicate that the PI3K activation of cyclin D1 involves Akt and IκB, consist with studies demonstrating that PI3K/Akt can activate NF-κB signaling [32]. The constitutively active mut IKKαCA(S176/180E), induced the cyclin D1 promoter 4-fold (Fig. 3a), while the constitutively active mutant IKKβ (IKKβCA) decreased promoter activity (see below) To determine the IKKα responsive element in the cyc D1 promoter, we first examined elements contributing to basal enhancer activity in the cyclin D1 promoter, whi contains several known transcription factor binding sites (eg. CRE, Tcf and NF-κB), that convey cell-type ba activity [33]. Point mutation of the CRE site or the Tcf site reduced basal cyclin D1 promoter activity by 20% a 55%, respectively (Fig. 3b). In contrast, mutation of the NF-κB binding site enhanced basal level activity (data shown), suggests that the NF-κB is a negative regulator of cyclin D1 in DU145 cells. Since the CRE and Tcf s together contribute to the majority of the cyclin D1 promoter activity in DU145 cells, we assessed the role of th elements in the activation of the cyclin D1 promoter by IKKα. Mutation of either of these sites abolished inducti of cyclin D1 by IKKαCA (Fig. 3c) (mutation of the NF-κB site had no effect on activation by IKKαCA(shown)). IKKαCA also induced TOP-LUC (a reporter construct that contains multimerized sequences identical the cyclin D1 Tcf site and which reflects β-catenin activity), 3-fold, similar to the activation of the canonical NF- responsive sequences (3xRelLUC) (Fig. 3c). The divergent effects of IKKα and IKKβ (see below Fig. 4) on cyc D1 raises the possibility that these kinases may be engaged by distinct upstream activators. We investigated whet NIK served as an upstream MAPKKK. NIK induced 3xRelLUC 20-fold, as previously shown [34] while the cyclin was repressed by NIK (Fig 3d). Conversely, a dominant negative NIK (KK429/430AA) inhibited 3xRelLUC activated cyclin D1 (Fig 3d), suggesting that the MAPKKK(s) that regulate NF-κB and cyclin D1 activity distinct. Next we compared the activity of NIK in SW480 cells, which contain a truncated APC gene and, a result, have increased β-catenin levels and signaling. In SW480 cells, wt NIK induced 3xRelLUC activity, repressed Tcf reporter activity (Fig. 3e). Since NIK plays a critical role in TNFα-dependent induction of NF- activity [32], we examined the effect of TNFα on the 3xRel reporter plasmid and found that TNFα induced 3xRelL but repressed Tcf activity (Fig. 3f). It appears, therfore, that different IKK kinases regulate IKK activity, resulting distinct downsteam signaling events. Overexpression of wt APC inhibited Tcf reporter activity and overexpressi of a constitutively active β-catenin (β-catenin S37A) rescued the repression by APC (Fig. 3h), consistent w previous studies of Tcf reporter activity and induction of cyclin D1 by β-catenin in these cells [8]. Thus IK activates Tcf signaling and the *cyclin D1* gene through a mechanism that is distinct from the pathway by whi TNFα and NIK regulate NF-κB activity.

Next we examined the ability of IKKβ to regulate Tcf activity. Constitutively active IKKβ (IKKβC increased NF-κB reporter activity, but in contract to IKKα, β-catenin signaling was decreased by 80-90% (Fig. 4 Furthermore, a kinase dead IKKβ mutant did not affect β-catenin activity (Fig. 4a), indicating that intact kin function is necessary for IKKβ to affect β-catenin signaling. IKKβCA also decreased the activity of a known catenin target gene, the cyclin D1 promoter construct, by 90% in SW480 cells (-163CD1Luc contains the functio Tcf/LEF sites as well as CREB, AP-1, Sp1 and NF-κB sites) (Fig. 4b). In contrast, a cyclin D1 promoter wit mutated Tcf/Lef site but which retained the other regulatory elements, was not responsive to IKKβ (Fig. 4b). T Tcf sequence was also sufficient for repression by IKKβCA when coexpressed in keratinocytes (Fig. 4c). Toget these results indicate that IKKα activation has a direct effect on the activity of the IKK complex toward β-cate signaling.

IKKα associates with and phosphorylates β-catenin and increases β-catenin abundance.

Several lines of evidence suggest that IKKα and IKKβ fulfill distinct functions. For example, homozyg deletion of the *IKKα* and *IKKβ* genes results in distinct phenotypes [15, 17, 18, 35]. Futhermore, IKKβ has hig activity for the IκB proteins and has a more significant role in the NF-κB pathway in response to activation w TNFα and IL-1 than does IKKα [15, 16, 36]. To investigate the possible mechanisms responsible for these dive functions, we assessed the subcellular localization of IKKα and IKKβ as well as cell type expression patter Western blot analysis of nuclear and cytoplasmic extracts confirmed the differential localization of IKKα and IK in Cos-7 cells (Fig. 5a). SKBR3 and SW480 cells which are of epithelial derivation, expressed significantly m IKKα than IKKβ, whereas Jurkat lymphocytic cells, expressed similar levels of IKKα and β (not show We hypothesize that this differential localization of the two IKK's is relevant for the precise regulation of b nuclear and cytoplasmic functions of IκB and β-catenin. Since IKKαCA induced cyclin D1 and Tcf reporter activ in SW480 cells, we hypothesized that IKKα may regulate β-catenin abundance and/or phosphorylation. determine whether IKKα could directly interact with β-catenin, immunoprecipitation was performed in Cos-7 ce co-transfected with HA-tagged β-catenin and FLAG-tagged IKKα. β-catenin is present in IK immunoprecipitates and IKKα is also present in β-catenin immunoprecipitates (Fig. 5b). Endogenous associati between β-catenin and IKKα was demonstrated by reciprocal IP-western blotting in SW480 cells (Fig. 5c). The to β-catenin level, as well as the amount of a higher molecular weight form of β-catenin and the level of the β-cate S37A mutant, were all increased in cells co-expressing IKKαCA and β-catenin expresion vectors (Fig.5 consistent with a role for IKKα in regulating β-catenin phosphorylation and/or abundance. Point mutation of catenin at S33 to alanine abrogated the induction of the higher molecular weight form of β-catenin. In the prese of IKKαCA, β-catenin abundance is increased and migrated as two distinct bands, with the slower migrating spec likely representing either a phosphorylated or mono-ubiquitinated form [37]. A phospho-β-catenin antibo demonstrated an induction of phosphorylated β-catenin forms in the presence of IKKαCA and the loss of t phosphorylated higher molecular weight form of S33Aβ-catenin (Fig. 5e). Since GSK3β phosphorylation of catenin results in a reduction of β-catenin levels, the increase in β-catenin levels by IKKαCA suggest that IK regulation of β-catenin is distinct from GSK3β. Consistent with findings in cultured cells, we found that β-cate fusion proteins were efficient substrates for phosphorylation by IKKα *in vitro* and the minimal region involved t N-terminal portion between aa 30 and 55 (Fig. 5f). IKKα bound to and phosphorylated β-catenin *in vitro* with efficiency that is similar to that of IκB (not shown). The findings that IKKα phosphorylates β-catenin and t IKKαCA increases Tcf activity and β-catenin abundance, suggests that β-catenin phosphorylation by IK contributes to a novel pathway of β-catenin-mediated Tcf-dependent gene transcription (Fig. 5g).

Figure Legends

FIG. 1. The PI3-kinase induction regulates cyclin D1 and S-phase entry. (A). Western blot analysis of mo embryo fibroblasts (MEFs) from cyclin D1 wild type (CD1wt) or CD1$^{-/-}$ mice and (B) DU145 cells, were treat with serum either with or without the PI3K inhibitor LY294002. Western blot was performed for cyclin D1, Ak total ERK and cyclin E. (B). FACS and (D) apopotosis analyses were performed of serum released CD1wt and C $^{/-}$ MEFs or (E) NIH3T3 cells treated either with vehicle or LY294002 (XμM).

FIG. 2. The cyclin D1 promoter is induced by PI3K (A). DU145 cells were transfected with the cyclin promoter luciferase reporter plasmid (-1745 CD1LUC) and the p110α-CAAX expression plasmid in 10% or (0.5% serum. The -fold induction of luciferase reporter activity is shown for 9 separate experiments as mean ± S throughout. (B). The effect of the p110α kinase dead mutant (p110α-CAAX-KD) or (C) constitutively acti p110α-K227E mutant on -1745 CD1LUC was assessed. (D) The effect of the p110α-CAAX expression plas was asessed in conjunction with reporter plasmids for cyclin A, cyclin E, and the luiferase reporter s pA_3LUC a pGL_3LUC.

FIG. 3. PI3K-induction of cyclin D1 involves IKKα, IκB. (A). The p110α-CAAX induced cyclin D1 promo activity was inhibited by LY294002 (from xmM to x mM) in either the presence or (B) absence of p110α-CA (C). p110α-CAAX induced cyclin D1 promoter activity was reduced by dominant negative inhibitors of PI (schematic) or by (D). coexpression of RacN17 or AktDN (K179M) but was not reduced by addition of chemi inhibitors of the MAPK pathways. The IκBSR and PDTC (xmm) are inhibitors of NFκB signaling. (F). The NF- luciferase reporter gene was induced by p110α-CAAX and inhibited by (G). LY294002 in a dose-depend manner.

FIG. 4. The IKKα, but not IKKβ pathway induces cyclin D1. (A). The p110-CAAX induced activity of th 1745 CD1LUC reporter was reduced by co-expression of the IKKα KM (K54M) or IKKαDN (S176/A) mutant. (The IKKα inhibitory mutants reduced cyclin D1 basal activity 50-to 60%. (C). The IKKαCA(S176E) mut activated the cyclin D1 promoter (D), requiring the CRE and TCF sites. (D). Schematic representation of the cyc D1 promoter, with the sequences homologous to CRE and Tcf binding sites indicated. (E). The IKKαCA(S176 induced the heterologous reporter encoding the Tcf site and the NF-κB reporter. (F). The IKK activating kin NIK, induced NF-κB reporter activity, but repressed the cyclin D1 promoter. The NIKDN(AA) inhiited the NF- reporter and induced the cyclin D1 promoter, indicating the IKKα kinase inducing cyclin D1 is not NIK.(G) SW4 cells were co-transfected with 0.1 μg APC and 0.1 μg β-catenin S37A in addition to TOPLUC and Renilla. A decreasesd β-catenin signaling by 80%. β-catenin S37A inhibited the ability of APC to decrease β-catenin signali (H) 3xRelLUC or FOPLUC reporters were coexpressed in SW480 cells with either NIKwt or (I) treated w TNFα. The data are mean ± SEM

FIG. 5. IKKβ induces NFκB but inhibits TCF activity. (A). Substantially more IKKα than IKKβ was found the nuclear extracts (NE) of Cos-7 cells. Internal controls for (nuclear) TFIIB and cytoplasmic markers are sho (B). The constitutively active IKKβCA(SE) inhibited activity of the TOP-LUC reporter and induced activity of t NF-κB reporter (3xRelLUC). (C). The cyclin D1 promoter was repressed by IKKβCA(SE) and deletion of the site abrogated repression. (D). The kinase inactive mutation of IKKα or IKKβ abrogated *trans*regulation of the site. (E). The IKKβCA(SE) inhiibited Tcf-LUC activity in SKBR3 and (F) human epidermal keratinocytes inhibited NFκB-reporter activity.

Fig. 6. IKKα associates with and phosphorylates β-catenin and increases β-catenin abundance. (A). SW4 cells were transfected with the constitutively active IKKα (IKKαCA(S/E)) and -1745 CD1LUC reporter or T

LUC. Fold induction is shown compared with equal amounts of empty expression vector cassette. (B). The effect the proteosome inhibitor ALLN on the IKKαCA(S/E) induced activity of TOP-LUC shows a 2-to 3-fold increa (C) Immunoprecipitation studies show that β-catenin associates with IKKα when either protein is precipitated fr cells transfected with Flag-IKKα and HA-β-catenin. (D). In cultured SW480 cells endogenous associations found between IKKα and β-catenin by immunoprecipitation western blot analysis. (E) The IKKαCA(S/ expression plamsid was coexpressed in cells transfected with either wild type or mutants of (S33A, S37A) of catenin. Western blotting shows an increase the total amount of β-catenin, including a higher molecular wei form (2 arrows). The S33Aβ-catenin shows no increase in the amount of the he high molecular weight form. (Western blot with phosphospecific β-catenin antibodies confirms the presence of phosphorylated β-cate including the higher molecular weight form, and the failure of this complex to form with the S33A β-catenin muta (G). GST-β–catenin fusion proteins were usied in IP-kinase assay with IKKα. The phosphorylated forms bcatenin are shown, together with the coomassie stained gel of the fusion proteins. (H). Model by which IK phosphorylates β-catenin at S33 to induce Tcf signaling of target genes including cyclin D1 .

Figure 1

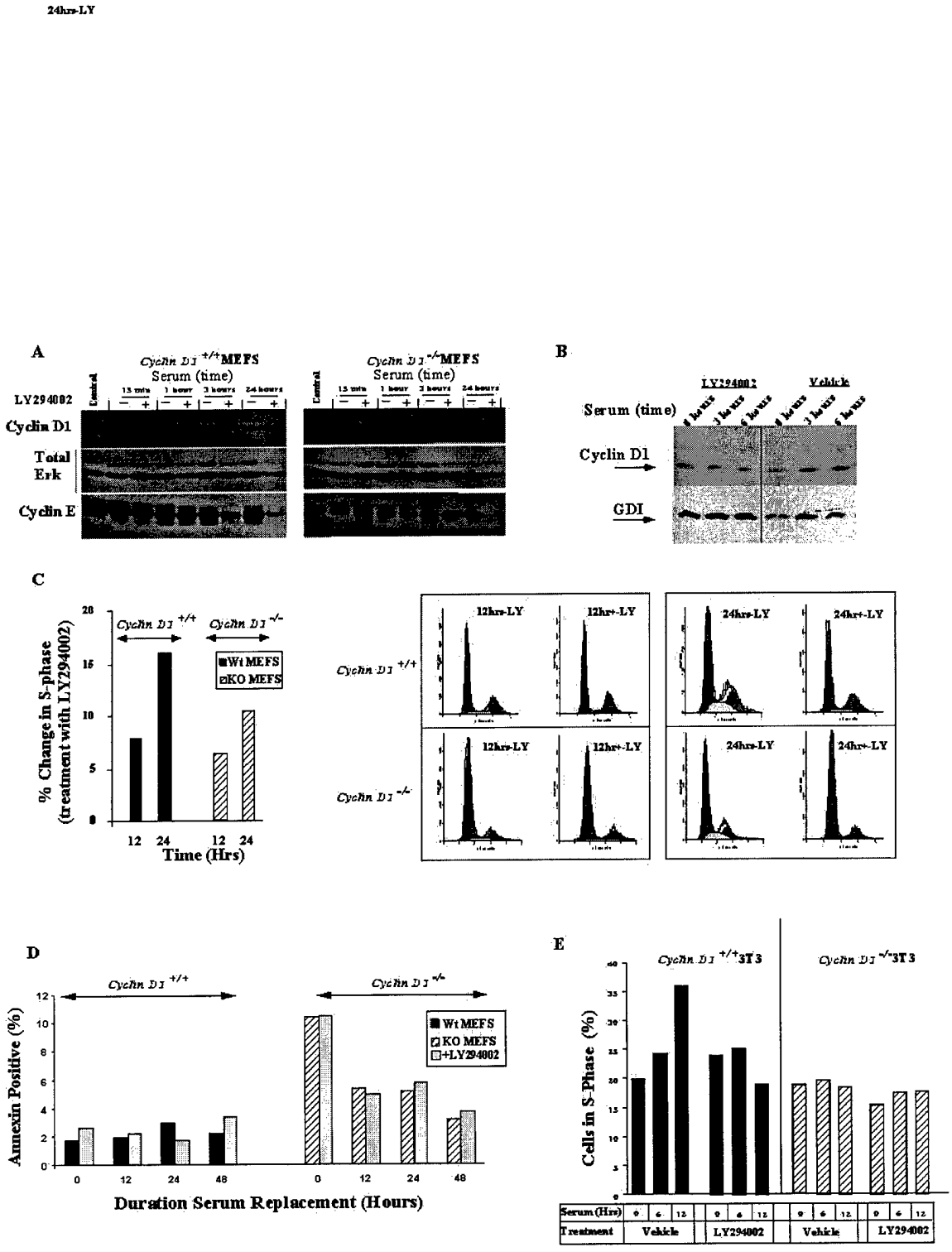
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Figure 2

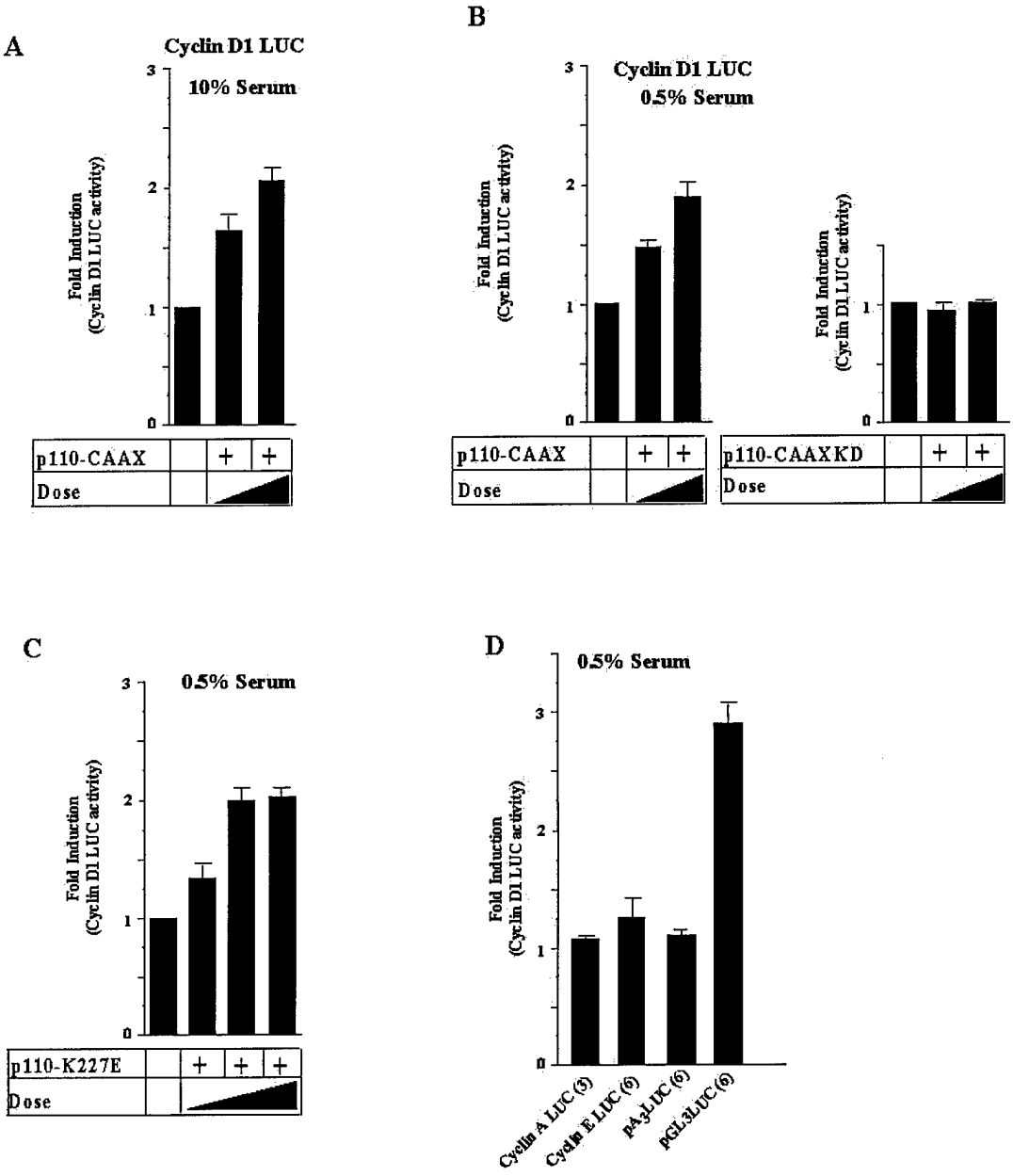
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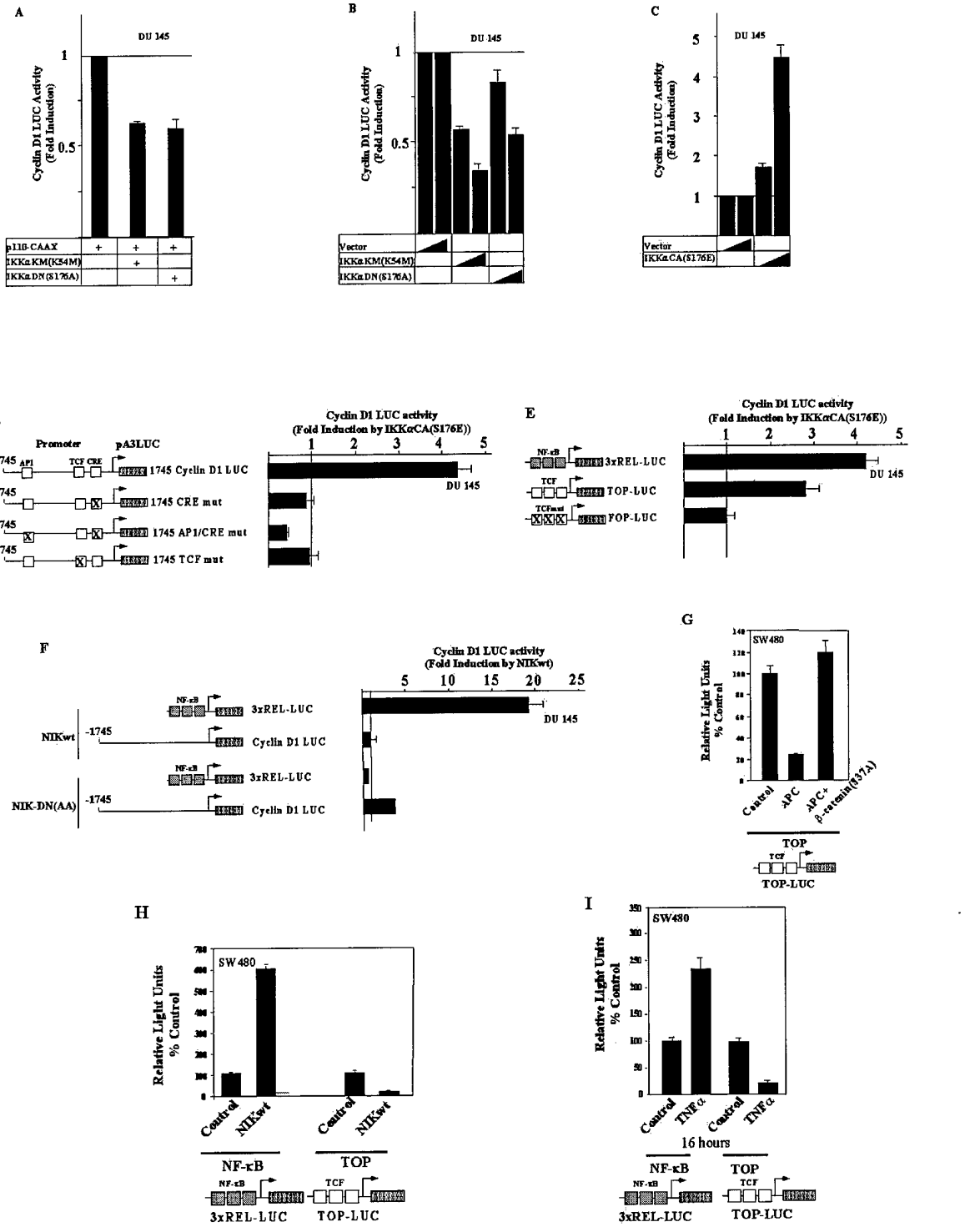
Figure 3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Cyclin D1 LUC Activity (Fold Induction)
Vector
IKKα CA(S176E)
D
Promoter
pA3LUC
-1745 AP1 TCF CRE 1745 Cyclin D1 LUC
-1745 1745 CRE mut
-1745 1745 AP1/CRE mut
-1745 1745 TCF mut
Cyclin D1 LUC activity (Fold Induction by IKKαCA(S176E))
DU 145
E
Cyclin D1 LUC activity (Fold Induction by IKKαCA(S176E))
3xREL-LUC
TOP-LUC
FOP-LUC
DU 145
F
Cyclin D1 LUC activity (Fold Induction by NIKwt)
NIKwt
NIK-DN(AA)
3xREL-LUC
Cyclin D1 LUC
DU 145
G
SW480
Relative Light Units % Control
Control
APC
APC+ β-catenin(S37A)
TOP
TOP-LUC
H
SW480
Relative Light Units % Control
Control
NIKwt
NF-κB
TOP
3xREL-LUC
TOP-LUC
I
SW480
Relative Light Units % Control
Control
TNFα
16 hours
NF-κB
TOP
3xREL-LUC
TOP-LUC

Figure 4

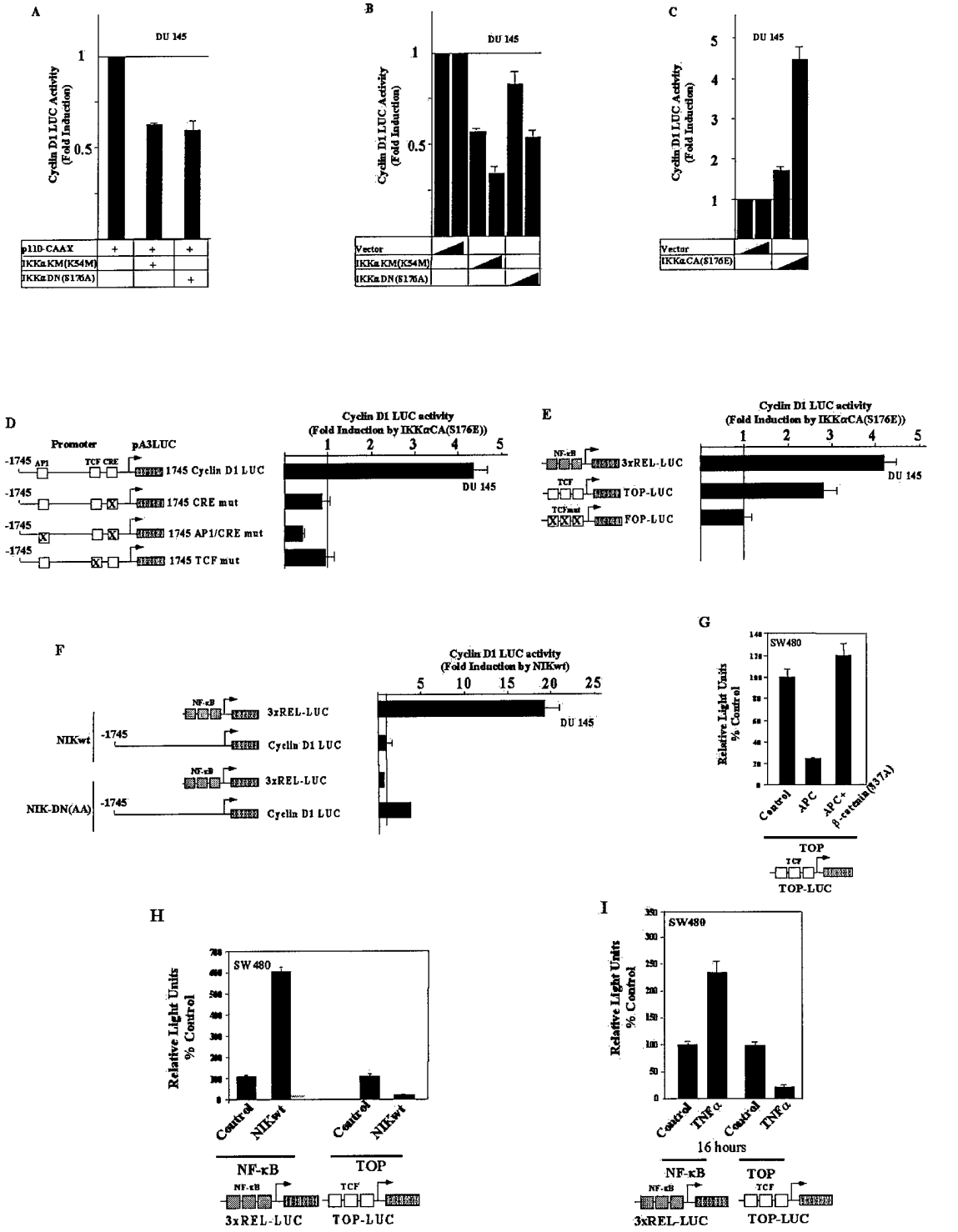

A
DU 145
Cyclin D1 LUC Activity (Fold Induction)
p110-CAAX
IKKαKM(K54M)
IKKαDN(S176A)
B
DU 145
Cyclin D1 LUC Activity (Fold Induction)
Vector
IKKαKM(K54M)
IKKαDN(S176A)
C
DU 145
Cyclin D1 LUC Activity (Fold Induction)
Vector
IKKαCA(S176E)
D
Promoter
pA3LUC
-1745
AP1
TCF CRE
1745 Cyclin D1 LUC
1745 CRE mut
1745 AP1/CRE mut
1745 TCF mut
Cyclin D1 LUC activity (Fold Induction by IKKαCA(S176E))
DU 145
E
Cyclin D1 LUC activity (Fold Induction by IKKαCA(S176E))
3xREL-LUC
TOP-LUC
FOP-LUC
DU 145
F
Cyclin D1 LUC activity (Fold Induction by NIKwt)
NIKwt
NIK-DN(AA)
3xREL-LUC
Cyclin D1 LUC
DU 145
G
SW480
Relative Light Units % Control
Control
APC
APC+ β-catenin(S37A)
TOP
TOP-LUC
H
SW480
Relative Light Units % Control
Control
NIKwt
NF-κB
TOP
3xREL-LUC
TOP-LUC
I
SW480
Relative Light Units % Control
Control
TNFα
16 hours
NF-κB
TOP
3xREL-LUC
TOP-LUC

Figure 5

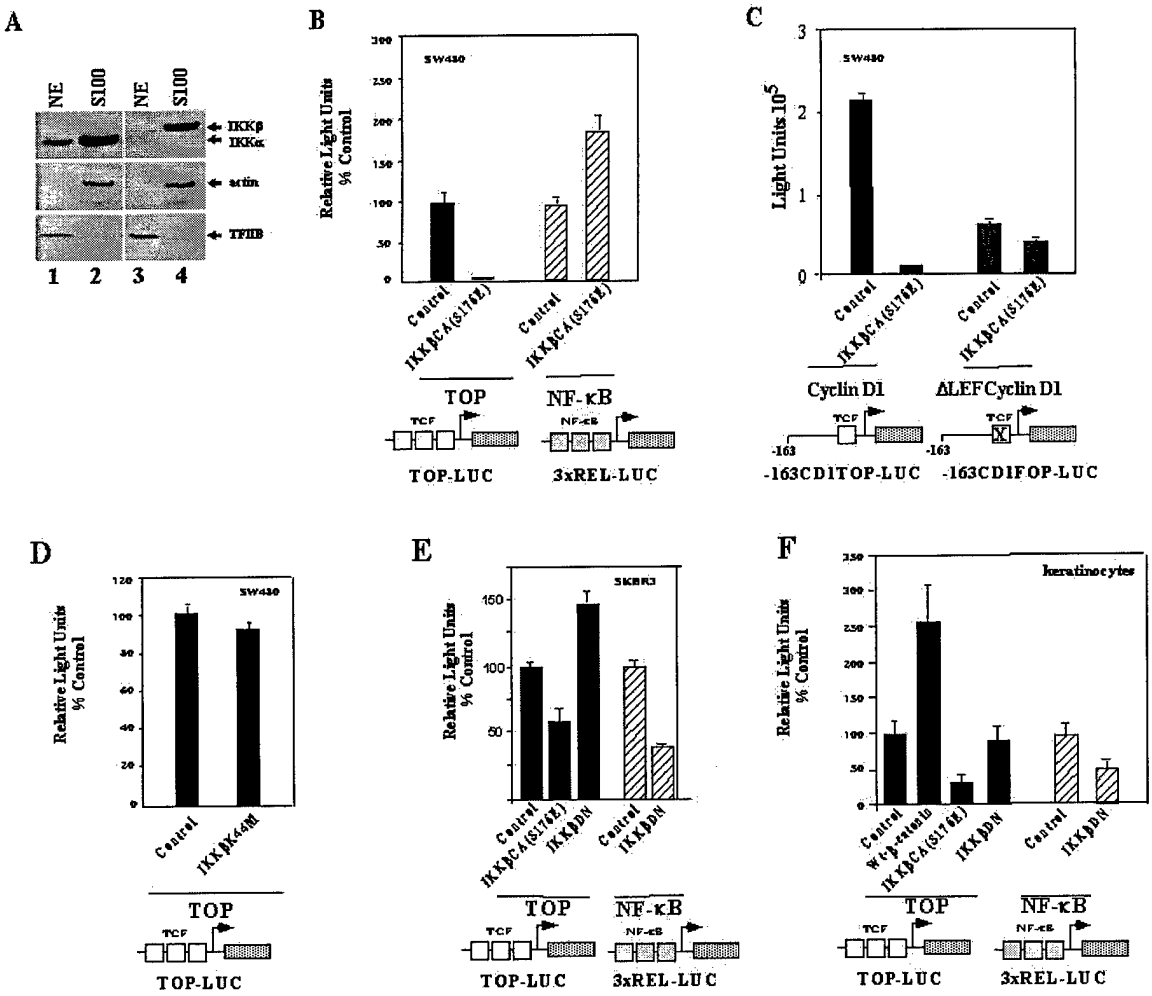
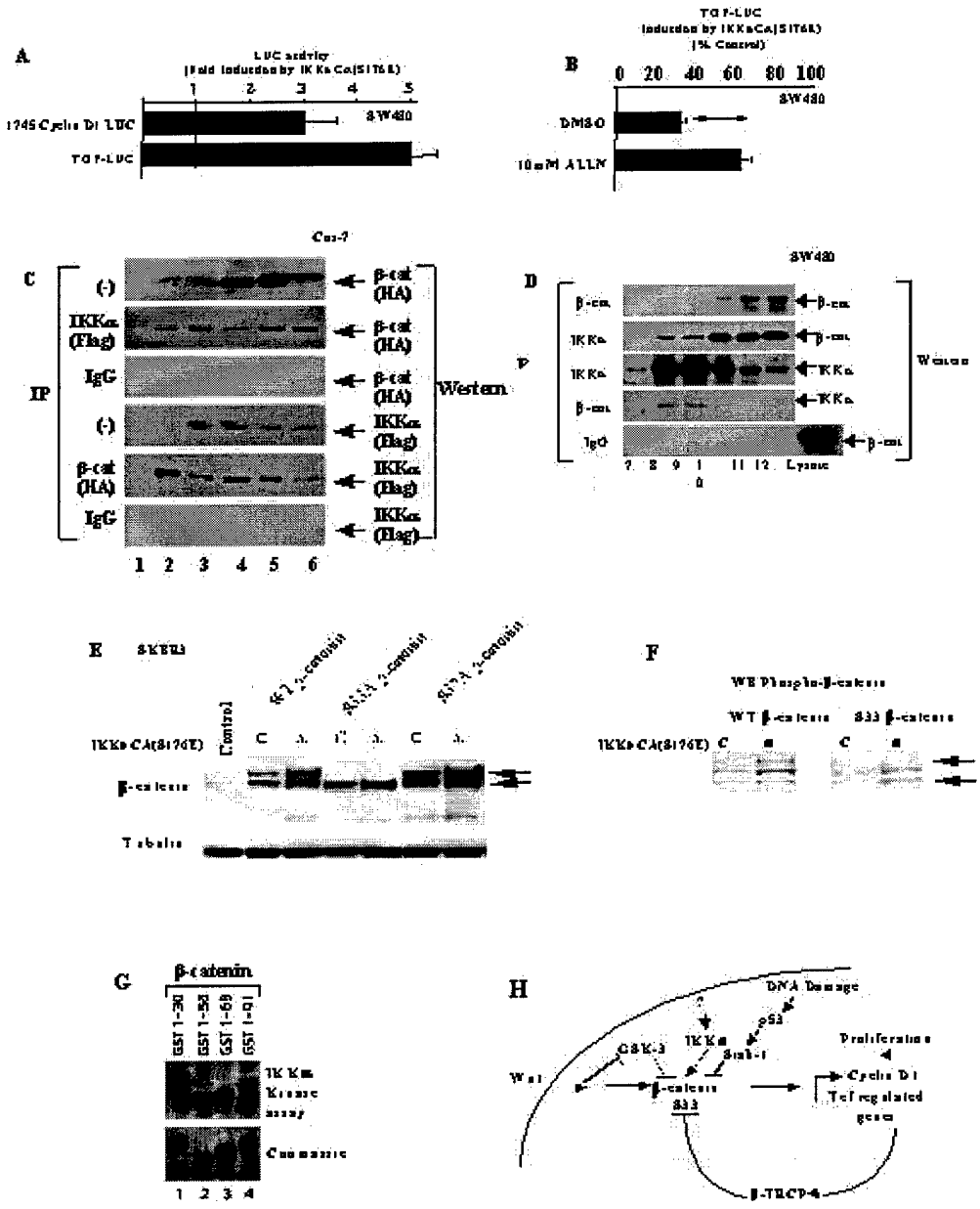

A
NE
S100
NE
S100
IKKβ
IKKα
actin
TFIIB
1 2 3 4
B
SW480
Relative Light Units % Control
Control
IKKβCA(S176E)
Control
IKKβCA(S176E)
TOP
NF-κB
TCF
NF-κB
TOP-LUC
3xREL-LUC
C
SW480
Light Units 10^5
Control
IKKβCA(S176E)
Control
IKKβCA(S176E)
Cyclin D1
ΔLEFCyclin D1
TCF
TCF
-163
-163
-163CD1TOP-LUC
-163CD1FOP-LUC
D
SW480
Relative Light Units % Control
Control
IKKβK44M
TOP
TCF
TOP-LUC
E
SKBR3
Relative Light Units % Control
Control
IKKβCA(S176E)
IKKβDN
Control
IKKβDN
TOP
NF-κB
TCF
NF-κB
TOP-LUC
3xREL-LUC
F
keratinocytes
Relative Light Units % Control
Control
Wt-β-catenin
IKKβCA(S176E)
IKKβDN
Control
IKKβDN
TOP
NF-κB
TCF
NF-κB
TOP-LUC
3xREL-LUC

Figure 6


A
LUC activity
(Fold Induction by IKKα CA(S176E))
1 2 3 4 5
1745 Cyclin D1 LUC
SW480
TCF-LUC
B
TCF-LUC
Induction by IKKαCA(S176E)
(% Control)
0 20 40 60 80 100
SW480
DMSO
10mM ALLN
C
Co-I
(-)
IKKα (Flag)
IgG
(-)
β-cat (HA)
IgG
β-cat (HA)
IKKα (Flag)
IP
Western
1 2 3 4 5 6
D
SW480
β-cat.
IKKα
IKKα
β-cat.
IgG
IP
Western
Lysate
E
SKBR3
Control
β-catenin
Tubulin
F
WB Phospho-β-catenin
WT β-catenin
S33 β-catenin
IKKα CA(S176E)
G
β-catenin
GST 1-30
GST 1-53
GST 1-69
GST 1-91
IK Kα Kinase assay
Coomassie
1 2 3 4
H
DNA Damage
ATM
p53
IKKα
Siah-1
GSK-3
Wnt
β-catenin
S33
Proliferation
Cyclin D1
Tcf regulated genes
β-TRCP-1

Key Research Accomplishments:

1. Cyclin D1 is required for PI3K-dependent S-phase entry in primary cells.
2. PI3K-induction of cyclin D1 is dependent upon IKKα.
3. IKKα, but not IKKβ induces cyclin D1 through β-catenin/Tcf
4. IKKα associates with and phosphorylates β-catenin and increases β-catenin abundance.

Reportable outcomes:

IKKα activates Tcf signaling through an interaction with and phosphorylation of β-catenin. C Albanese*, CR. Jarrett†, F Attiga†, D Joyce*, J Hughes*, J Hulit*, T Sakamaki*, K Wu*, M D'Amico*, M Fu*, A Ben-Ze'ev‡, C Lamberti¶, K-M Lin¶, RB. Gaynor¶, SW. Byers† and RG. Pestell. Submitted

Conclusions:

This study demonstrated a novel pathway involving IKKα in the β-catenin-mediated regulation of activity. Endogenous IKKα was found in association with β-catenin in cultured cells while a constitutively acti form of IKKα increased both β-catenin abundance and phosphorylation and induced Tcf-dependent transcripti This effect of IKKα on β-catenin was dependent, at least in part, on the S33 residue of β-catenin. Tightly regulat activity of the β-catenin/Tcf pathway is crucial for normal development. Aberrant activation of the Tcf pathw contributes to the development of a variety of human cancers, including colon, breast and prostate cancer. Mutati in components of the Wnt-signaling pathway are commonly observed in human cancer and result in t accumulation of β-catenin and activation of Tcf/Lef target genes. Wnt family ligands and frizzled family recept define one important mechanism that can induce β-catenin/Tcf signaling. In addition, suppressor screens *Drosophila* have identified *Dpresenilin* as a target of *Armadillo* (a homolog of β-catenin) and cell-adhesi dependent pathway involving the integrin linked kinase (ILK) can also control β-catenin levels and activity. T *cyclin D1* gene which plays a critical role in oncogenic signaling pathways, is regulated (via its Tcf site) by seve of these component pathways which regulate β-catenin/Tcf signaling. Thus, in addition to this study, in which t *cyclin D1* gene was induced by IKKα, previous studies have demonstrated the activation of the *cyclin D1* gene stable mutants of β-catenin, Wnt-1, ILK and repression by presenilin 1. Cyclin D1 and β-catenin over-express correlates with poor prognosis in human breast cancer suggesting a role for cyclin D1 in β-catenin/Tcf signaling a transformation. Understanding the relative impact of each of the pathways regulating β-catenin/Tcf activity and th effectors of cellular growth is fundamental to understanding dysregulation of this pathway in cancer.